FEBS Letters 583 (2009) 3225–3229

FEBS Letters
journal homepage: www.FEBSLetters.org

The cytosolic subunit p67^{phox} of the NADPH-oxidase complex does not bind NADPH

Laura Baciou [a], Marie Erard [a], Marie-Claire Dagher [b], Tania Bizouarn [a,*]

[a] Laboratoire de Chimie-Physique, UMR 8000-CNRS, Université Paris-Sud, Orsay, France
[b] Centre Diagnostic et Recherche CGD, TIM-C TheREx, UMR 5525-CNRS, Université Joseph-Fourier, CHU Grenoble, Grenoble, France

ARTICLE INFO

Article history:
Received 21 August 2009
Revised 3 September 2009
Accepted 6 September 2009
Available online 12 September 2009

Edited by Miguel De la Rosa

Keywords:
NADPH-oxidase
NADPH-binding
Tryptophan fluorescence
NADPH-dialdehyde
p67^{phox}

ABSTRACT

The NADPH-oxidase of phagocytic cells is a multicomponent enzyme that generates superoxide. It comprises a membrane flavocytochrome b_{558} and four cytosolic proteins; p67^{phox}, p47^{phox}, p40^{phox} and Rac. The NADPH-binding site of this complex was shown to be located on the flavocytochrome b_{558}. However, a number of studies have suggested the presence of another site on the p67^{phox} subunit which is the key activating component. Using several approaches like tryptophan quenching fluorescence measurement, inhibition by 2′,3′-dialdehyde NADPH, and free/bound NADPH concentration measurements, we demonstrate that no NADPH binds on p67^{phox}, thus definitively solving the controversy on the number and location of the NADPH-binding sites on this complex.

1. Introduction

Human neutrophils play an essential role in the inflammatory response to kill invading pathogens. Upon stimulation, neutrophils exhibit a burst of cyanide-insensitive oxygen consumption accompanied by a NADPH–dependent production of superoxide anions ($O_2^{\cdot -}$), precursors of toxic reactive oxygen metabolites. $O_2^{\cdot -}$ production is catalysed by a membrane-bound electron transfer complex named NADPH-oxidase (for review see [1,2]). Dysfunction of the phagocyte NADPH-oxidase leads to severe human pathologies like the chronic granulomatous disease (CGD).

The NADPH-oxidase is a highly regulated enzyme complex, dormant in resting cells and active upon cell stimulation. The activation occurs *via* the phosphorylation of cytosolic regulatory components p47^{phox}, p67^{phox}, p40^{phox} and their translocation to the transmembrane heterodimer, the flavocytochrome b_{558} (Cytb_{558}) in the presence of the small G protein Rac1/2. The Cytb_{558} is composed of two subunits, p22^{phox} and gp91^{phox}. The cytosolic cofactors p47^{phox}, p67^{phox} are absolutely required for the enzymatic activity in vivo [1,3]. In vitro, the, so-called "cell-free system", comprised of p67^{phox}, p47^{phox}, Rac in its GTP bound form and the Cytb_{558}, is able to produce $O_2^{\cdot -}$ upon addition of NADPH (for review see [4]). In vitro, p47^{phox} can be omitted if the concentration of p67^{phox} is raised up to several micromolar [5]. The canonical view is that the NADPH-oxidase catalyses hydride transfer from NADPH to FAD and successive electron transfers from FAD to the catalytic centre (two hemes). The NADPH-binding site was first proposed to be located on another component than Cytb_{558}, hypothesis based on studies of fractions isolated from X-linked-CGD patient's neutrophils (lacking the gp91^{phox} subunit). In these fractions, NADPH-binding proteins were detected and the oxidase activation property (i.e. $O_2^{\cdot -}$ production) was inhibited by covalent binding of NADPH analogs (NADPH-dialdehyde) [6]. In addition, it was found that the oxidase cytosolic subunits bound to a 2′,5′-ADP (a NADPH analog) agarose gel and could be eluted with ATP, GTP and NADPH solutions [7]. Curiously, after labelling of the neutrophil fractions with radioactive nucleotides, the radioactivity was found on various size proteins ranging from 32 to 66 kDa [6,8–10].

In the early 90s, the publication of the first hint of the presence of the catalytic NADPH (and FAD) binding site on the Cytb_{558} strongly suggesting that the C-terminus of gp91^{phox} is a member of the FNR family of reductase [11] clearly weakened the previous hypothesis. This proposal was strengthened by labelling studies from other groups showing a predominant labelling by a radioactive NADPH analog on a glycosylated membrane-bound protein of about 80–100 kDa [12,13] and by functional studies [14] showing that NADPH dependent $O_2^{\cdot -}$ production can be elicited in a cell-free system containing Cytb_{558} in the total absence of cytosolic

Abbreviations: CGD, chronic granulomatous disease; GST, glutathione-S-transferase; MF, neutrophil membrane fraction

* Corresponding author. Address: Laboratoire de Chimie-Physique, UMR 8000-CNRS, Université Paris-Sud, 91405 Orsay Cedex, France. Fax: +33 1 69156188.
E-mail address: bizouarn@lcp.u-psud.fr (T. Bizouarn).

0014-5793/$36.00
doi:10.1016/j.febslet.2009.09.011

components. However, more recent studies on recombinant p67^{phox} gave controversial results. Using radioactive NADPH-dialdehyde for protein labelling and tryptophan fluorescence quenching measurements, it was proposed that the recombinant p67^{phox} protein contains a NADPH-binding site essential for enzyme activity, with a K_d close to 7 μM [15].

Altogether, these results maintain a doubt on the presence of multiple NADPH-binding site in the oxidase complex, as underlined in several recent reviews [2,16,17]. An assessment of the binding site on p67^{phox} subunit is essential since it would be the primary catalytic event within the enzyme, all other steps depending on substrate binding. The location of the nucleotide binding site is reanalysed here on bovine and human p67^{phox} recombinant proteins in order to eliminate a possible species peculiarity and with different approaches to allow the detection of a large range of K_d value from 10^{-7} to 10^{-4} M.

2. Materials and methods

2.1. Materials

All the chemicals were obtained from Sigma–Aldrich; the Q-Sepharose Fast-Flow (FF), DEAE Sepharose-FF, SP-Sepharose-HP, Glutathione-Sepharose-4B gels were from GE-Healthcare-Bioscience; the His-Select Nickel Affinity gel was from Sigma–Aldrich.

2.2. Recombinant protein productions and purifications

All the constructs used to produce the recombinant proteins from the NADPH-oxidase are listed in Table S1 (Supplementary data). Except the His-p67^{phox} protein which was expressed in *Escherichia coli* BL21(DE3)pLysS and induced only 6 h (30 °C), all recombinant proteins were induced in BL21(DE3) overnight at 30 °C. The glutathione-S-transferase (GST)-tagged proteins (p67^{phox} and Rac1) were purified on Q-Sepharose-FF chromatography followed by a Glutathione-Sepharose-4B affinity column (elution with 50 mM Tris, 10 mM reduced glutathione, pH 8.0). The His-p67^{phox} was purified as above except that it was purified on a Nickel Affinity gel (elution with 150 mM imidazole). The His-p47^{phox} was purified through the SP-Sepharose chromatography and purified further through a Nickel Affinity gel.

The fusion-tags could be removed by the use of either thrombin or Factor-Xa. Since the human and bovine p67^{phox} proteins have an additional cleavage site for these endoproteases, only the bovine p67^{phox} was subjected to limited digestion with protease Factor-Xa (2 h at room temperature; 40 units of protease/mg of protein).

Protein concentrations were estimated using the Bicinchoninic acid protein assay with BSA as standard. All isolated proteins were subjected to 10% BisTris-NuPAGE SDS gels (Invitrogen), stained with Coomassie Brilliant Blue (Fig. 1).

The intact nicotinamide nucleotide transhydrogenase and its NADH-binding domain were purified as described in [18,19].

2.3. Purification of membrane fraction from neutrophils (MF)

The membrane fraction was obtained after bovine neutrophils purification from blood as described in [20]. The yield was between 5 and 20 mg of membrane protein from 10 l of blood.

2.4. Tryptophan fluorescence spectroscopy measurement

The tryptophan fluorescence signals of bovine and human p67^{phox} were recorded in a 1 × 1 cm quartz cuvette on a Spex-Fluorolog1681 spectrofluorimeter at 25 °C with an excitation and emission wavelengths of 280 nm and 340 nm, respectively. The

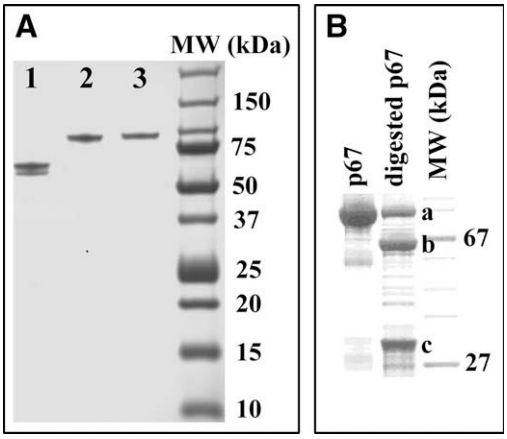

Fig. 1. SDS–PAGE electrophoresis of recombinant purified cytosolic p67^{phox} and GST-cleaved bovine GST-His-p67^{phox}. (A) Each lane was loaded with 0.5–1.5 μg of human His-p67^{phox} (lane 1), human GST-p67^{phox} (lane 2) and bovine GST-His-p67^{phox} (lane 3), molecular weight marker (dual-color, Biorad). (B) Undigested and Factor-Xa digested bovine GST–His-p67^{phox} (a: GST-tagged p67^{phox}, b: p67^{phox} and c: GST-tag) and the molecular weight marker (Ozyme).

titrations were achieved by successive additions of 5 μl aliquots of 3 mM NADPH (or NADH when indicated). A titration in identical conditions was performed on a bovine serum albumin (BSA) sample (0.5 μM), a well-known non-NADPH-binding protein. This was used to correct the fluorescence signals from inner filtering effect [21] and dilution. To validate this method, negative and positive controls were performed with tryptophanyl solution (8 μM) titrated with NADPH and NADH-binding domain of transhydrogenase (2.5 μM) with NADH, respectively. Indeed, transhydrogenase has a K_d value for NADH (20 μM) in the same range as the one expected for the NADPH-oxidase complex [18].

2.5. Estimation of K_d by unbound and bound NADPH concentration measurements

Four milligrams of p67^{phox} were mixed with 2 ml of Glutathione-Sepharose gel for 1 h. The gel was washed twice briefly by centrifugation (at 10 000 rpm) with PBS buffer to remove the unbound proteins and then distributed in four tubes. In each tube, NADPH was added to final concentrations of 0 μM, 36 μM, 71 μM, 135 μM (in 0.5 ml of PBS). After 10 min incubation, the tubes were centrifuged as previously. At this stage, the protein (15–20 μM) is located at the bottom of the tube bound onto the gel and the free NADPH concentration can be determined from an aliquot taken on top of the tube. The NADPH fluorescence of the diluted supernatant (v:v 1:20) was recorded (λ_{ex} = 340 nm, λ_{em} = 460 nm). Control experiments, in the absence of proteins, were performed in parallel. The levels of fluorescence in the absence of NADPH as well as the concentration of the bound protein were checked. A positive control, showing that dinucleotides can bind to gel-trapped proteins was realised with a His-tagged nicotinamide nucleotide transhydrogenase bound on Nickel resin. We found that all the binding sites were occupied at the NADPH concentrations used in these experiments, which is in agreement with a K_d for NADPH of 1 μM.

It should be noted that some purified protein preparations displayed an intrinsic NADPH oxidation activity without addition of any oxidant. When this reaction was observed, it occurred extremely slowly. However, it could cause artefacts in the estimation of free NADPH concentration essentially because of the high level of protein concentration used (15–20 μM). We evaluated this undesirable NADPH oxidation by measuring spectrophotometrically the NADPH absorption decrease at 340 nm for several hours

at room temperature by mixing purified cytosolic protein (200 μg/ml PBS) with NADPH (60 μM) for each protein preparation. The preparations presenting this activity were systematically discarded for the binding experiments described in this report.

2.6. Preparation of NADPH-dialdehyde and protein binding procedure

The reduction of $NADP^+$-dialdehyde was obtained as described in [22] with some modifications. One millimolar of the $NADP^+$ analog was incubated with 2 mM isocitrate and 2 units of NADP-isocitrate dehydrogenase for 3 h in Tris buffer (50 mM Tris, 4 mM $MgCl_2$, pH 8.0) at room temperature. The sample was then loaded on a 5 ml DEAE-Sepharose column and the NADPH-dialdehyde was eluted with a 0–1 M NaCl gradient of 50 ml in the same buffer. The purity and the concentration of the NADPH-dialdehyde was determined by measuring its absorbance at 340 nm and 260 nm (ε = 6.22 cm^{-1} mM^{-1} and 15.5 cm^{-1} mM^{-1}, respectively [22]). The covalent binding of the analog to $p67^{phox}$ or to the membrane fraction was performed by mixing the protein solution (0.2–0.6 mg/ml) with NADPH-dialdehyde (30, 73 and 110 μM), in the presence of $NaCNBH_3$ (500 μM). The mixture was incubated for 20 h at 5 °C. The samples were then used for NADPH-oxidase activity measurements. As control, the same protocol was used in the absence of NADPH-dialdehyde and in the presence of NADPH-dialdehyde and NADPH (2 mM).

Table 1
Estimation of V_{max} and K_m of NADPH for $O_2^{\cdot-}$ production catalysed by the NADPH-oxidase complex reconstituted either with bovine or human $p67^{phox}$. Prior to NADPH and cytochrome c addition, MF (18 μg), $p67^{phox}$ (15 μg), $p47^{phox}$ (16 μg), RacQ61L (8 μg) and arachidonate (40 μg) are incubated 5 min in 1 mL PBS buffer and 10 mM $MgSO_4$.

	V_{max} (μmol $O_2^{\cdot-}$/min/mg membrane prot)	K_m (μM)
Bovine GST–His-$p67^{phox}$	0.34 ± 0.02	54 ± 9
Human GST-$p67^{phox}$	0.42 ± 0.04	62 ± 15
Human His-$p67^{phox}$	0.35 ± 0.02	31 ± 8

2.7. Cell-free NADPH-oxidase activity assays

The $O_2^{\cdot-}$ production initiated by the addition of NADPH was measured at 550 nm as the rate of superoxide dismutase-inhibited cytochrome c reduction (ε = 21 mM^{-1} cm^{-1} [23]), according to [24] and as described in the legend. Michaelis-Menten constant (K_m) and V_{max} were determined from a titration of the activity as a function of NADPH concentration (5–300 μM).

3. Results

3.1. Comparison of NADPH-oxidase activities between the bovine and the human recombinant $p67^{phox}$

The sequence comparison of the bovine and human $p67^{phox}$ shows a very high similarity (88% of identity) [25]. We measured the rate of superoxide anion production (V_{max}) in cross-species complex to estimate the relevance of the specific interactions of cytosolic subunits (bovine or human) with bovine membrane fractions (Table 1). No significant difference in the V_{max} values was observed in cell-free assays containing either the human or the bovine recombinant $p67^{phox}$ protein, whatever the tag present at the N-terminus of the recombinant protein. The obtained V_{max} and K_m values are in good agreement with the literature (25–50 μM) [8,15,26]. Our results suggest that the human GST-$p67^{phox}$ leads to a NADPH-oxidase complex as efficient as the one obtained in the presence of the bovine GST–His-$p67^{phox}$ protein and that no species dependent interactions are essential to build an efficient NADPH-oxidase complex. In addition, these results indicate that neither the GST fusion protein nor the His-tag in N-terminus of cytosolic proteins alter superoxide production, binding of NADPH to the complex or protein interactions for correct assembly.

3.2. NADPH-binding studies on $p67^{phox}$ recombinant proteins

In the absorption and fluorescence spectra of protein preparations, no peak characteristic to NADPH have been detected (data not shown). So, there is no tightly bound NADPH to $p67^{phox}$ with

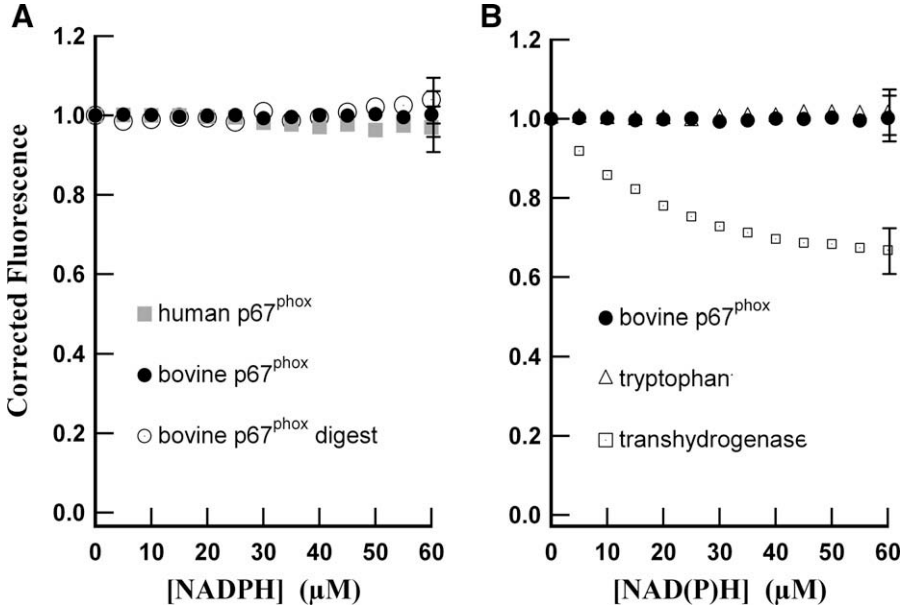

Fig. 2. The relative quenching of tryptophan fluorescence by NADPH (A) human $p67^{phox}$ (0.13 mg), bovine $p67^{phox}$ or digested bovine $p67^{phox}$ (0.16 mg) and (B) bovine $p67^{phox}$ (0.15 mg), tryptophan (8 μM) and NADH-binding domain of transhydrogenase (0.3 mg) (all sample diluted in PBS buffer, final volume 3 mL). The data are normalized to the tryptophan fluorescence level before the addition of NAD(P)H and corrected of the inner filtering effects using the BSA titrations. Same titrations (A) were obtained for three different $p67^{phox}$ preparations.

a dissociation constant value (K_d) less than 0.1 μM. In order to determine higher NADPH dissociation constant values, two methods have been chosen. First, we followed the quenching of the endogenous tryptophan fluorescence at 340 nm by Förster resonant energy transfer (FRET) to bound NADPH [15] upon excitation at 280 nm (Fig. 2). For either bovine or human p67^{phox}, the fluorescence intensity does not vary upon addition of NADPH (Fig. 2A). For the bovine p67^{phox}, similar titrations were also observed after digestion of the fusion domain by Factor-Xa (Fig. 2A). As positive control, the NADH-binding domain of nicotinamide nucleotide transhydrogenase displays a significant tryptophan fluorescence quenching by NADH titration (Fig. 2B). By this method, the K_d value of this protein was estimated to be 20 μM in agreement with the literature [18]. In summary, all these titrations show that no NADPH binding was detected on p67^{phox} whenever the GST-tag is present or not.

The second approach consisted in mixing NADPH with p67^{phox} trapped on affinity gel before measuring the free NADPH concentration remaining in solution. The results, presented Fig. 3, indicate that the concentration of free NADPH was the same with and without proteins whatever the NADPH concentration. Similar results were obtained with human p67^{phox} preparations (data not shown). This experiment reinforces the conclusion drawn from the titrations of tryptophan fluorescence quenching by NADPH.

3.3. Binding of NADPH-dialdehyde on p67^{phox} and on the membrane fraction of neutrophils

The two methods presented above are not sensitive enough to measure higher dissociation constant values (>100 μM). In order to probe this range of K_d, an analog of NADPH, NADPH-dialdehyde, associated with sodium cyanoborohydride was used. This analog irreversibly binds to the recognition site of NADPH by a covalent reaction and therefore should inhibit the NADPH-oxidase activity. As shown in Fig. 4, no inhibition of superoxide production was found when human or bovine p67^{phox} were previously treated with the inhibitor. In contrast, when the membrane fraction was treated with increased inhibitor concentrations, the activities decrease by 30–90%. These results suggest that the NADPH-dialdehyde was

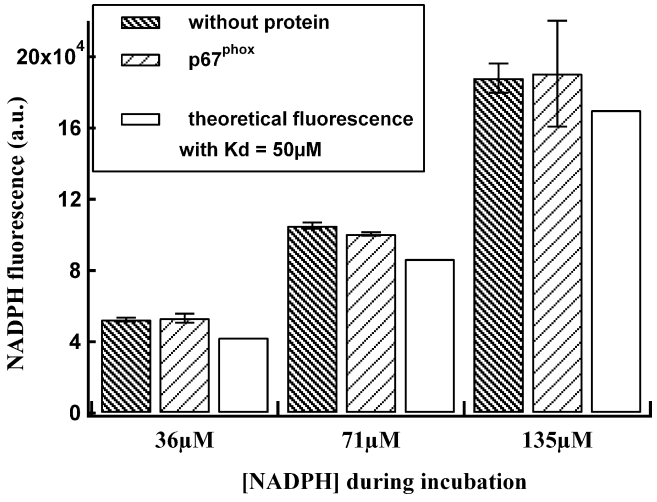

Fig. 3. Binding measurements of NADPH on bovine p67^{phox}. The proteins bound to Glutathione-Sepharose were incubated with various concentrations of NADPH (36, 71 and 135 μM). After centrifugation, the free NADPH concentration was estimated from the supernatant by fluorescence measurements. Experiments without proteins were realised in parallel as negative controls. As guidance, the theoretical level of fluorescence expected for a K_d value of 50 μM and a concentration of protein of 20 μM is indicated as white bars.

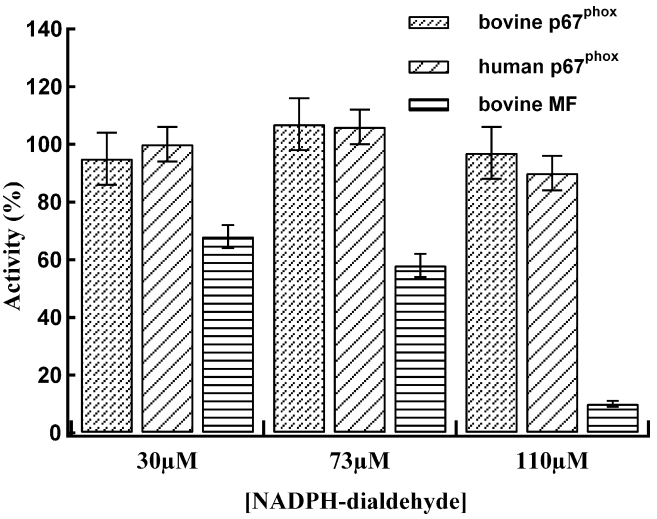

Fig. 4. Effect of NADPH-dialdehyde treatment of p67^{phox} and MF on the $O_2^{\cdot-}$ production rate. For a given protein, NADPH-oxidase activity is given as the percentage of the activity obtained in the presence vs. absence of NADPH-dialdehyde. The data is the average of three independent measurements (standard deviation less than 10%). The rate of $O_2^{\cdot-}$ production with the untreated proteins was consistently around 0.3 μmol $O_2^{\cdot-}$/min/mg membrane protein.

able to react with a catalytic binding site on the membrane fraction while not on p67^{phox}. The NADPH specificity of this reaction was assessed by performing the experiment in the presence of 2 mM NADPH. In that case, no inhibition of activity was observed except when a 110 μM inhibitor concentration was used, with 60% activity remaining. This is in good agreement with the degree of inhibition usually found with these concentrations of nucleotide [22,27].

These results clearly demonstrate that NADPH binds to the membrane fraction containing Cytb$_{558}$ but not to the p67^{phox} protein.

4. Discussion

As mentioned earlier, NADPH is the electron donor for the one electron reduction of molecular oxygen to superoxide anion by the NADPH-oxidase, thus a NADPH-binding site must be present in the catalytic core. Over the years, the NADPH-binding site has been identified on several candidates, some of them in the cytosolic fraction [8,28] and more specifically on p67^{phox} [15]. In parallel, several evidences have shown that the Cytb_{558} was able to functionally bind FAD and NADPH. Therefore, two hypotheses have been formulated, either p67^{phox} participates directly in electron transfer between NADPH and FAD [29], or there would be a site on p67^{phox}, additional to the one on gp91^{phox}, with an unknown regulatory function [15]. This site would be located in the N-terminal part of p67^{phox} (1–210 amino acids), critical for oxidase activity [15]. For further functional studies, especially for a detailed analysis of the electron transfer between the redox prosthetic groups within gp91^{phox}, it was crucial to ascertain the number and the location of the NADPH-binding site(s). Thus, we carried out experiments that cover a large range of K_d values, from 10^{-7} to 10^{-4} M whose reliability and reproducibility of the results have been improved with the systematic use of recombinant cytosolic proteins. Indeed, it should be noted that most of the previous studies were carried out on non-purified cytosolic fractions of neutrophils, which are likely to contain a number of NADPH-binding proteins that could be responsible for the discrepancies observed. With all these cautions, the experiments depicted in this work lead to the conclusion that NADPH has no specific binding site on either bo-

vine or human p67^{phox}. From the structural point of view, the C-terminus of gp91^{phox} could be modelized with a nucleotide binding motif [30], whereas the available crystallographic 3D structures of the N-terminus of p67^{phox} do not show such a motif [31].

During our systematic analysis of the binding properties of p67^{phox}, several constructs have been used with either a GST-tag or a His-tag or both. It should be noted that the lack of alteration of NADPH-oxidase activity with a tag as large as GST (26 kDa) is promising for functional studies with other tags like fluorescent protein homologues of the Green Fluorescent Protein (GFP, 23 kDa).

In conclusion, the role of p67^{phox} is not to bring NADPH to the complex in order to initiate electron transfer. However, this does not exclude a function in the control of NADPH-binding affinity through conformational changes induced in the Cytb_{558} structure through a direct interaction between the two proteins and/or in the regulation of the electron flow and more precisely of flavin reduction by NADPH.

Acknowledgements

We are very grateful to Dr. M. Quinn (Montana State University, USA) for providing plasmids carrying the bovine p47^{phox} and p67^{phox} cDNAs, Dr. A. Hall (University College, London) for providing plasmid carrying Rac1 cDNA and Dr G. Gacon (Institut Cochin, Paris, France) for cDNA of p67^{phox} in pGADGH (Clontech). We thank Dr. F. Lederer for helpful discussion. This work was supported by the ANRjc Grant from the French National Agency for Research [JCJC06-137200].

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.09.011.

References

[1] Cross, A.R. and Segal, A.W. (2004) The NADPH oxidase of professional phagocytes–prototype of the NOX electron transport chain systems. Biochim. Biophys. Acta 1657, 1–22.
[2] Quinn, M.T. and Gauss, K.A. (2004) Structure and regulation of the neutrophil respiratory burst oxidase: comparison with nonphagocyte oxidases. J. Leukoc. Biol. 76, 760–781.
[3] Vignais, P.V. (2002) The superoxide-generating NADPH oxidase: structural aspects and activation mechanism. Cell. Mol. Life Sci. 59, 1428–1459.
[4] Dagher, M.C. and Pick, E. (2007) Opening the black box: lessons from cell-free systems on the phagocyte NADPH-oxidase. Biochimie 89, 1123–1132.
[5] Freeman, J.L. and Lambeth, J.D. (1996) NADPH oxidase activity is independent of p47phox in vitro. J. Biol. Chem. 271, 22578–22582.
[6] Umei, T., Takeshige, K. and Minakami, S. (1987) NADPH-binding component of the superoxide-generating oxidase in unstimulated neutrophils and the neutrophils from the patients with chronic granulomatous disease. Biochem. J. 243, 467–472.
[7] Sha'ag, D. and Pick, E. (1988) Macrophage-derived superoxide-generating NADPH oxidase in an amphiphile-activated, cell-free system; partial purification of the cytosolic component and evidence that it may contain the NADPH binding site. Biochim. Biophys. Acta 952, 213–219.
[8] Umei, T., Babior, B.M., Curnutte, J.T. and Smith, R.M. (1991) Identification of the NADPH-binding subunit of the respiratory burst oxidase. J. Biol. Chem. 266, 6019–6022.
[9] Ge, F. and Guillory, R.J. (1994) NADPH-binding protein of the neutrophil superoxide-generating oxidase of guinea pigs. Proc. Natl. Acad. Sci. USA 91, 8622–8626.
[10] Smith, R.M., Curnutte, J.T. and Babior, B.M. (1989) Affinity labeling of the cytosolic and membrane components of the respiratory burst oxidase by the 2′,3′-dialdehyde derivative of NADPH. Evidence for a cytosolic location of the nucleotide-binding site in the resting cell. J. Biol. Chem. 264, 1958–1962.
[11] Segal, A.W. et al. (1992) Cytochrome b-245 is a flavocytochrome containing FAD and the NADPH-binding site of the microbicidal oxidase of phagocytes. Biochem. J. 284 (Pt 3), 781–788.
[12] Ravel, P. and Lederer, F. (1993) Affinity-labeling of an NADPH-binding site on the heavy subunit of flavocytochrome b558 in particulate NADPH oxidase from activated human neutrophils. Biochem. Biophys. Res. Commun. 196, 543–552.
[13] Doussière, J., Brandolin, G., Derrien, V. and Vignais, P.V. (1993) Critical assessment of the presence of an NADPH binding site on neutrophil cytochrome b558 by photoaffinity and immunochemical labeling. Biochemistry 32, 8880–8887.
[14] Koshkin, V. and Pick, E. (1993) Generation of superoxide by purified and relipidated cytochrome b559 in the absence of cytosolic activators. FEBS Lett. 327, 57–62.
[15] Dang, P.M., Johnson, J.L. and Babior, B.M. (2000) Binding of nicotinamide adenine dinucleotide phosphate to the tetratricopeptide repeat domains at the N-terminus of p67PHOX, a subunit of the leukocyte nicotinamide adenine dinucleotide phosphate oxidase. Biochemistry 39, 3069–3075.
[16] Sheppard, F.R., Kelher, M.R., Moore, E.E., McLaughlin, N.J., Banerjee, A. and Silliman, C.C. (2005) Structural organization of the neutrophil NADPH oxidase: phosphorylation and translocation during priming and activation. J. Leukoc. Biol. 78, 1025–1042.
[17] Groemping, Y. and Rittinger, K. (2005) Activation and assembly of the NADPH oxidase: a structural perspective. Biochem. J. 386, 401–416.
[18] Bizouarn, T., Diggle, C., Quirk, P.G., Grimley, R.L., Cotton, N.P., Thomas, C.M. and Jackson, J.B. (1996) Interaction of nucleotides with the NAD(H)-binding domain of the proton-translocating transhydrogenase of *Rhodospirillum rubrum*. J. Biol. Chem. 271, 10103–10108.
[19] Hu, X., Zhang, J., Fjellstrom, O., Bizouarn, T. and Rydstrom, J. (1999) Site-directed mutagenesis of charged and potentially proton-carrying residues in the beta subunit of the proton-translocating nicotinamide nucleotide transhydrogenase from *Escherichia coli*. Characterization of the beta H91, beta D392, and beta K424 mutants. Biochemistry 38, 1652–1658.
[20] Morel, F., Doussiere, J., Stasia, M.J. and Vignais, P.V. (1985) The respiratory burst of bovine neutrophils. Role of a b type cytochrome and coenzyme specificity. Eur. J. Biochem. 152, 669–679.
[21] Li, B. and Lin, S.X. (1996) Fluorescence-energy transfer in human estradiol 17 beta-dehydrogenase-NADPH complex and studies on the coenzyme binding. Eur. J. Biochem. 235, 180–186.
[22] Lark, R.H. and Colman, R.F. (1986) Reaction of the 2′,3′-dialdehyde derivative of NADPH at a nucleotide site of bovine liver glutamate dehydrogenase. J. Biol. Chem. 261, 10659–10666.
[23] van Gelder, B. and Slater, E.C. (1962) The extinction coefficient of cytochrome c. Biochim. Biophys. Acta 58, 593–595.
[24] Doussière, J. and Vignais, P.V. (1985) Purification and properties of an $O_2^{-\cdot}$-generating oxidase from bovine polymorphonuclear neutrophils. Biochemistry 24, 7231–7239.
[25] Bunger, P.L., Swain, S.D., Clements, M.K., Siemsen, D.W., Davis, A.R., Gauss, K.A. and Quinn, M.T. (2000) Cloning and expression of bovine p47-phox and p67-phox: comparison with the human and murine homologs. J. Leukoc. Biol. 67, 63–72.
[26] Babior, B.M. (1984) The respiratory burst of phagocytes. J. Clin. Invest. 73, 599–601.
[27] Umei, T., Takeshige, K. and Minakami, S. (1986) NADPH binding component of neutrophil superoxide-generating oxidase. J. Biol. Chem. 261, 5229–5232.
[28] Smith, R.M., Connor, J.A., Chen, L.M. and Babior, B.M. (1996) The cytosolic subunit p67phox contains an NADPH-binding site that participates in catalysis by the leukocyte NADPH oxidase. J. Clin. Invest. 98, 977–983.
[29] Dang, P.M., Babior, B.M. and Smith, R.M. (1999) NADPH dehydrogenase activity of p67PHOX, a cytosolic subunit of the leukocyte NADPH oxidase. Biochemistry 38, 5746–5753.
[30] Taylor, W.R., Jones, D.T. and Segal, A.W. (1993) A structural model for the nucleotide binding domains of the flavocytochrome b-245 beta-chain. Protein Sci. 2, 1675–1685.
[31] Lapouge, K., Smith, S.J., Walker, P.A., Gamblin, S.J., Smerdon, S.J. and Rittinger, K. (2000) Structure of the TPR domain of p67phox in complex with Rac.GTP. Mol. Cell 6, 899–907.